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(54) Title: METHODS OF SCREENING FOR THERAPEUTIC AGENTS USING NOVEL APOPTOSIS-MODULATING PROTEINS

(57) Abstract

The present invention provides methods to screen for anti-viral agents utilizing a novel family of apoptosis-modulating proteins.

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METHODS OF SCREENING FOR THERAPEUTIC AGENTS USING NOVEL APOPTOSIS-MODULATING PROTEINS

TECHNICAL FIELD

This invention relates to methods of screening for therapeutic agents using novel proteins with apoptosis-modulating activity.

BACKGROUND ART

Apoptosis is a normal physiologic process that leads to individual cell death. This process of programmed cell death is involved in a variety of normal and pathogenic biological events and can be induced by a number of unrelated stimuli. Changes in the biological regulation of apoptosis also occur during aging and are responsible for many of the conditions and diseases related to aging. Recent studies of apoptosis have implied that a common metabolic pathway leading to cell death may be initiated by a wide variety of signals, including hormones, serum growth factor deprivation, chemotherapeutic agents, ionizing radiation and infection by human immunodeficiency virus (HIV). Wyllie (1980) *Nature 284*:555-556; Kanter et al. (1984) *Biochem. Biophys. Res. Commun. 118*:392-399; Duke and Cohen (1986) *Lymphokine Res. 5*:289-299; Tomei et al. (1988) *Biochem. Biophys. Res. Commun. 155*:324-331; Kruman et al. (1991) *J. Cell. Physiol. 148*:267-273; Ameisen and Capron (1991) *Immunology Today 12*:102; and Sheppard and Ascher (1992) *J. AIDS 5*:143. Agents that modulate the biological control of apoptosis thus have therapeutic utility in a wide variety of conditions.

Apoptotic cell death is characterized by cellular shrinkage, chromatin condensation, cytoplasmic blebbing, increased membrane permeability and interchromosomal DNA cleavage. Kerr et al. (1992) *FASEB J. 6*:2450; and Cohen and Duke (1992) *Ann. Rev. Immunol. 10*:267. The blebs, small, membrane-encapsulated spheres that pinch off of the surface of apoptotic cells.

may continue to produce superoxide radicals which damage surrounding cell tissue and may be involved in inflammatory processes.

The Bcl-2 gene was discovered at the common chromosomal translocation site t(14:18) in follicular lymphomas and results in aberrant over-expression of bcl-2. Tsujimoto et al. (1984) *Science 226*:1097-1099; and Cleary et al. (1986) *Cell 47*:19-28. The normal function of bcl-2 is the prevention of apoptosis; unregulated expression of bcl-2 in B cells is thought to lead to increased numbers of proliferating B cells which may be a critical factor in the development of lymphoma. McDonnell and Korsmeyer (1991) *Nature 349*:254-256; and, for review see, Edgington (1993) *Bio/Tech. 11*:787-792. Bcl-2 is also capable of blocking of γ irradiation-induced cell death. Sentman et al. (1991) *Cell 67*:879-888; and Strassen (1991) *Cell 67*:889-899. It is now known that bcl-2 inhibits most types of apoptotic cell death and is thought to function by regulating an antioxidant pathway at sites of free radical generation. Hockenbery et al. (1993) *Cell 75*:241-251.

Apoptosis, a normal cellular event, can also be induced by pathological conditions and a variety of injuries. Apoptosis is involved in a wide variety of conditions including, but not limited to: cardiovascular disease; cancer regression; immunoregulation; viral diseases; anemia; neurological disorders; gastrointestinal disorders such as diarrhea and dysentery; diabetes; hair loss; rejection of organ transplants; prostate hypertrophy; obesity; ocular disorders; stress; and aging.

Bcl-2 belongs to a family of proteins of which some have been cloned and sequenced. Williams and Smith (1993) *Cell 74*:777-779. Various Bcl-2 members have the ability to associate with one another as heterodimers. Oltvai et al. (1993) *Cell 74*:609-619; and Sato et al. (1994) *Proc. Natl. Acad. Sci. USA 91*:9238-9242. Additionally, BHRF1 displays a 25% sequence identity to Bcl-2 (Cleary et al. (1986) *Cell 47*:19-28) and has been shown by gene transfer studies to protect B cells from apoptosis. Henderson et al. (1993) *Proc. Natl. Acad. Sci. USA 90*:8479-8483.

The herpesvirus family of viruses typically produce latent and recurrent infections. Herpesvirus genomes are composed of sequences with a short and a long region. Herpesvirus particles have a diameter from 180 nm to 200 nm. Many particles do not contain envelopes. Typically, the DNA is wrapped around an associated protein. The herpesvirus has a tendency to persist in a quiescent state for irregular periods of time.

All references cited herein, both supra and infra, are hereby incorporated by reference herein.

SUMMARY OF THE INVENTION

Methods of screening for pharmaceutical agents that stimulate, as well as pharmaceutical agents that inhibit Bak and Bak-2 protein activity levels are provided. The methods include combining a Bak protein and a viral protein under conditions in which they interact to form a test sample, exposing the test sample to a potential therapeutic agent and monitoring the interaction of the proteins. Potential therapeutic agents which disrupt the interaction compared to control test samples to which no agent has been added are selected for further study.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the Bak cDNA nucleotide sequence and amino acid sequence encoded thereby.

Figure 2 shows the sequence of the Bak-2 cDNA and flanking sequences and the corresponding predicted amino acid sequence of the Bak-2 protein.

Figure 3 shows the interactions of Bak and Flag-Bak (F-Bak) fusion proteins with Epstein-Barr virus BHRF-1 protein. In column A, lane 1 depicts the results obtained from *in vitro* co-translated proteins F-Bak/BHRF-1 and lane 2 depicts F-Bak/BHRF-1 proteins bound to anti-FLAG agarose. In column B, the lanes are the same with the exception that the Bak protein is Bak-2.

DISCLOSURE OF THE INVENTION

The present invention provides methods of screening for potential anti-viral therapeutic agents. The proteins encoded by nucleotide sequences encoding the novel bc1-2 homologs, Bak and Bak-2 proteins have been found to interact with the Epstein Barr Virus (EBV) protein BHRF1 indicating that Bak proteins contribute to the pathogenicity of the disease. BHRF1 is an EBV early lytic cycle protein. Pearson et al. (1987) *Virol. 160*:151-161. The invention encompasses methods containing the steps of exposing the Bak proteins and viral proteins, or functional portions thereof, to potential therapeutic agents and monitoring the interaction of the proteins. The invention further utilizes recombinant cells and transgenic animals expressing the cloned Bak or Bak-2 genes.

The cloning and analysis of Bak genes and proteins are described in detail in commonly owned WO application PCT/US94/13930. Bak genes and proteins are also described in Kiefer et al. (1995) *Nature 374*:736. The nucleotide and predicted amino acid residue sequences of Bak protein are shown in Figure 1; and those of Bak-2 are shown in Figure 2. Bak mRNA has been detected in a variety of human organs and tissues by Northern blot analysis. These organs include liver; heart; skeletal muscle; lung; kidney; and pancreas.

These references also disclose that the Bak proteins are capable of modulating apoptosis. In a lymphoblastoid cell line, expression of Bak protein was shown to decrease Fas-mediated apoptosis. In a mouse progenitor B cell line, FL5.12, Bak-2 protein and a derivative of Bak protein decrease IL-3-induced apoptosis whereas Bak protein increased apoptosis. Thus, depending on the cell type, the derivative of Bak protein, and the method of induction of apoptosis, apoptosis can be modulated in a highly specific manner by controlling the concentration of Bak proteins.

As used herein, the term "Bak gene(s)" refers to the nucleic acid molecules described herein and in PCT/US94/13930, "the Bak protein(s)" refers to the proteins encoded thereby. The nucleotides include, but are not limited to, the

cDNA and complementary DNA, genome-derived DNA and synthetic or semi-synthetic DNA or RNA. The nucleotide sequence of the Bak cDNA with the location of restriction endonuclease sites is shown in Figure 1.

The nucleotide sequence of Bak-2 cDNA, along with the predicted amino acid sequence of Bak-2 protein and the locations of restriction endonuclease recognition sites, is given in Figure 2. The Bak gene is on human chromosome 6 and the Bak-2 gene is on human chromosome 20. There is also a member of the family, Bak-3, which is on human chromosome 11. Bak-3 appears to be a pseudogene. Fluorescence *in situ* hybridization (FISH) indicated an approximate location of the Bak gene to be at 6p21-23.

The invention includes the use of modified Bak DNA sequences such as deletions, substitutions and additions particularly in the non-coding regions of genomic DNA. Such changes are useful to facilitate cloning and modify gene expression. Any DNA which encodes a portion of a Bak protein sufficient to bind to BHRF1 or any other suitable viral protein is suitable for use herein. As described below, various fusion proteins are suitable for use herein.

Various substitutions can be made within the coding region that either do not alter the amino acid residues encoded or result in conservatively substituted amino acid residues. Nucleotide substitutions that do not alter the amino acid residues encoded are useful for optimizing gene expression in different systems. Suitable substitutions are known to those of skill in the art and are made, for instance, to reflect preferred codon usage in the particular expression systems.

The invention encompasses the use of functionally equivalent variants and derivatives of Bak genes which may enhance, decrease or not significantly affect the properties of Bak proteins. For instance, changes in the DNA sequence that do not change the encoded amino acid sequence, as well as those that result in conservative substitutions of amino acid residues, one or a few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are those which will not significantly affect its properties.

Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. Any conservative amino acid substitution which does not significantly affect the properties of Bak proteins is encompassed by the present invention.

Techniques for nucleic acid manipulation useful for the practice of the present invention are described in a variety of references, including, but not limited to, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Vol. 1-3, eds. Sambrook *et al.*, Cold Spring Harbor Laboratory Press (1989); and *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, Greene Publishing and Wiley-Interscience: New York (1987) and periodic updates.

The coding regions of Bak genes can also be ligated into expression vectors capable of stably integrating into other cell types including but not limited to cardiomyocytes, neural cell lines such as GTI-7 and TNF sensitive cells such as the human colon adenocarcinoma cell line HT29 so as to provide a variety of assay systems to monitor the regulation of apoptosis by Bak proteins.

As used herein, "BHRF1" or "viral proteins" encompasses the full length EBV protein and portions or derivations thereof sufficient to bind to Bak proteins or portions or derivatives thereof. Such proteins include, but are not limited to, homologous proteins expressed by any virus, particularly various forms of herpes and herpes-like viruses, such as cytomegalovirus and varicella zoster.

The interaction between a Bak protein and viral protein such as BHRF-1 can be produced by adding purified proteins together. Preferably, however, the proteins are cotranscribed and translated under conditions that allow protein-protein interactions. Co-translation can be performed in vitro or in vivo in whole cells expressing native or recombinant Bak proteins and viral proteins. Any suitable recombinant expression vectors may be used. The Bak proteins can also

be separately translated and then combined under conditions that allow for protein-protein interactions.

Methods of monitoring protein interactions are known in the art, any method is suitable for use herein. Preferably, co-precipitation is used. The ability of an antibody to precipitate one of the proteins or an immunological tag fused thereto is used to immunoprecipitate the protein and the immunoprecipitate is monitored for the presence of both proteins. Methods of co-precipitation are known in the art and are described in the examples below. Any other method in the art is suitable for use herein, including, but not limited to, protein interactive trapping, such as GST fusion protein immobilization on glutathione columns and, ELISA. Immunological tags are often incorporated into fusion proteins and including, for instance, FLAG, hemagglutinin and glutathione-S transferase.

Purification or isolation of Bak proteins expressed either by the recombinant DNA or from biological sources such as tissues can be accomplished by any method known in the art. Protein purification methods are known in the art. Generally, substantially purified proteins are those which are free of other, contaminating cellular substances, particularly proteins. Preferably, the purified Bak proteins are more than eighty percent pure and, most preferably, more than ninety-five percent pure. For clinical use as described below, the Bak proteins are preferably highly purified, at least about ninety-nine percent pure, and free of pyrogens and other contaminants.

Suitable methods of protein purification are known in the art and include, but are not limited to, affinity chromatography, immunoaffinity chromatography, size exclusion chromatography, HPLC and FPLC. Any purification scheme that does not result in substantial degradation of the protein is suitable for use herein.

As used herein, "Bak proteins" includes functionally equivalent variants thereof which do not significantly affect their properties and variants which retain the same overall amino acid sequence but which have enhanced or decreased activity. For instance, conservative substitutions of amino acid residues, one or a

few amino acid deletions or additions, and substitution of amino acid residues by amino acid analogs are within the scope of the invention.

Amino acid residues which can be conservatively substituted for one another include but are not limited to: glycine/alanine; valine/isoleucine/leucine; asparagine/glutamine; aspartic acid/glutamic acid; serine/threonine; lysine/arginine; and phenylalanine/tyrosine. Any conservative amino acid substitution which does not significantly affect the properties of Bak proteins is encompassed by the present invention.

Suitable antibodies for use herein are generated by using the Bak proteins as an antigen or, preferably, peptides encompassing the Bak protein regions that lack substantial homology to the other gene products of the bcl family. Antibodies to the viral proteins are also suitable for use herein. Methods of detecting proteins using antibodies and of generating antibodies using proteins or synthetic peptides are known in the art and are not described in detail herein.

Screening for therapeutically effective agents is done by exposing the Bak protein and the viral protein to such agents which may directly or indirectly affect the interaction between a Bak protein and a viral protein. Suitable potential therapeutic agents include, but are not limited to, any pharmaceutical agent such as cytokines, small molecule drugs, cell-permeable small molecule drugs, hormones, combinations of interleukins, lectins and other stimulating agents, e.g., PMA, LPS, bispecific antibodies, peptide mimetics, antisense oligonucleotides and other agents which modify cellular functions or protein expression.

The proteins are added together or co-expressed, exposed to such agents at physiologically effective concentrations, and the interaction thereof is measured relative to a control not exposed to such agents. Those biological modifiers which decrease the interaction between a Bak protein and a viral protein relative to a control are selected for further study of their anti-viral activity.

As previously shown, overexpressed Bak proteins protect EBV-transformed B cells from apoptosis following serum withdrawal or anti-Fas

treatment. PCT/US94/13930. These results indicate that a Bak-BHRF1 interaction exists whereby BHFR1 not only neutralizes the normally apoptotic effect of Bak protein, but additionally induces a protective activity. Alternatively, propagation of cells transfected with the Bak cDNA might select for cells that are expressing high levels of BHRF1 or other EBV encoded anti-apoptotic proteins. This could lead to an anti-apoptotic response upon subjecting the cells to an apoptosis signal such as serum withdrawal. Example 2 shows that *in vitro* translated Flag-Bak (epitope tagged) and BHRF1 can be coprecipitated with an antibody that recognizes the Flag epitope indicating that Bak proteins and BHRF1 interact directly with one another.

The following examples are provided to illustrate but not limit the present invention. Unless otherwise specified, all cloning techniques were essentially as described by Sambrook et al. (1989) and all reagents were used according to the manufacturer's instructions.

Example 1

Expression of Recombinant Bak Gene

In order to express the recombinant Bak gene in the baculovirus system, the Bak cDNA generated as described in PCT/US94/13930 was used to generate a novel Bak vector, by PCR, using primers from the 3' and 5' flanking regions of the gene which contain restriction sites to facilitate cloning. The plasmids were sequenced by the dideoxy terminator method (Sanger *et al.*, 1977) using sequencing kits (USB, Sequenase version 2.0) and internal primers. This was to confirm that no mutations resulted from PCR.

A clone was used to generate recombinant viruses by *in vivo* homologous recombination between the overlapping sequences of the plasmid and AcNPV wild type baculovirus. After 48 hours post-transfection in insect Spodoptera frugiperda clone 9 (SF9) cells, the recombinant viruses were collected, identified by PCR and further purified. Standard procedures for selection, screening and propagation of recombinant baculovirus were performed in accordance with the

manufacturer's instructions (Invitrogen). The molecular mass, on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), of the protein produced in the baculovirus system was compared with the predicted molecular mass of Bak protein according to the amino-acid sequence.

In addition, similar clones can be expressed in any expression system known in the art including, but not limited to, bacterial, yeast, insect and mammalian. A suitable yeast intracellular expression system is described by Barr et al. (1992) *Transgenesis* ed. JAH Murray, (Wiley and Sons) pp. 55-79.

The Bak gene coding sequence was excised and introduced into plasmids pCEP7, pREP7 and pcDNA3 (Invitrogen) at compatible restriction enzyme sites. pCEP7 was generated by removing the RSV 3'-LTR of pREP7 with *XbaI*/*Asp*718, and substituting the CMV promoter from pCEP4 (Invitrogen). 25 μg of each Bak-containing plasmid was electroporated into the B lymphoblastoid cell line WIL-2, and stable hygromycin resistant transformants or G418 resistant transformants (pcDNA3 constructs) expressing Bak were selected.

Example 2

Bak proteins interact with Epstein-Barr Virus encoded BHRF1 protein

BHRF1 cDNA was amplified by RT-PCR from WI-L2 mRNA using standard PCR protocol according to the instructions of the manufacturers of the PCR kit and thermal cycler (Perkin Elmer Cetus). The Flag-Bak and Flag-Bak-2 cDNAs were generated by RT-PCR as above from Bak and Bak-2/pcDNA3 plasmid templates but included the 24 base Flag encoding sequence 5'-GAC TAC AAG GAC GAC GAT GAC AAG-3' in the sense primer. This resulted in a cDNA encoding N-terminal Flag-Bak and Flag-Bak-2 fusion proteins that could be recognized and purified by the anti-Flag M2 antibody (Kodak-IBI). The cDNAs were ligated into the pcDNA3 vector which is under the control of the CMV and T7 RNA polymerase promoter. The Flag-Bak and BHRF1 plasmids or Flag-Bak-2 and BHRF1 plasmids were then cotranscribed and cotranslated using

the TnT coupled reticulocyte lysate system according to manufacturer's instructions (Promega).

Briefly, 0.5-1.0 μg of the two circular plasmids were simultaneously transcribed and translated in 50 μL of TnT lysate for 90 minutes at 32°C. After translation, 20 μL of lysate was mixed with 20 μL of 2X PBS plus 40 μL of anti-Flag M2 affinity gel (Kodak) and incubated with gentle rocking overnight at 4°C. Immunoprecipitates were collected by centrifugation in an Eppendorf microfuge at 1500 rpm for 15 minutes at 4°C. Pellets were washed 4 times with 1.5 mL PBS and after the final wash were resuspended in 30 μL of SDS-PAGE sample buffer. The samples were then analyzed by SDS-PAGE on a 18% polyacrylamide gel. Gels were fixed with 10% glacial acetic acid, dried and exposed to X-ray film overnight at room temperature.

As shown in Figure 3, Flag-Bak and BHRF1 as well as Flag-Bak-2 and BHRF1 were efficiently cotranscribed and cotranslated (lanes 1). Clearly, the anti-Flag M2 antibody effectively coprecipitates Flag-Bak and BHRF1 or Flag-Bak-2 and BHRF1 (lanes 2). This demonstrates that BHRF1 interacts with both Bak and Bak-2 proteins *in vitro* and suggests that such interactions occur *in vivo* resulting in the modulation of apoptosis. Interactions of Bak proteins with viral proteins are likely to have evolved to allow viral replication or latency to proceed in the absence of apoptotic death of the host cell. Interference, therefore, in these interactions represents an important new strategy for the design of novel antiviral agents. Similarly, malignant cells derived from transformation by viruses such as EBV would also be amenable to diagnosis or therapy with these agents.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

CLAIMS

We claim:

1. A method for screening potential anti-viral therapeutic agents comprising the steps of:

(a) combining a Bak protein and a viral protein under conditions in which they interact, to form a test sample;

(b) exposing the test sample to a potential therapeutic agent and;

(c) monitoring the interaction of the Bak protein and the viral protein; wherein, a potential therapeutic agent is selected for further study when it disrupts the interaction compared to a control test sample to which no potential therapeutic agent has been added.

2. The method according to claim 1, wherein the potential therapeutic agent is selected from the group consisting of any pharmaceutical agent, cytokines, small molecule drugs, cell-permeable small molecule drugs, hormones, combinations of interleukins, lectins and other stimulating agents e.g. PMA, LPS, bispecific antibodies, peptide mimetics, antisense oligonucleotides and other agents which modify cellular functions or protein expression.

3. The method according to claim 1, wherein the Bak protein is selected from the group consisting of Bak, Bak-2, portions thereof sufficient to effect binding to a viral protein and fusion proteins thereof containing a portion thereof sufficient to effect binding to a viral protein.

4. The method according to claim 3, wherein the fusion protein is selected from the group consisting of epitope-tagged Bak protein and epitope-tagged Bak-2 protein.

5. The method according to claim 1, wherein the viral protein is selected from the group consisting of Epstein Barr Virus BHRF1 protein, other EBV encoded anti-apoptotic proteins and proteins homologous to BHRF1 expressed by herpes viruses and herpes-like viruses.

6. The method according to claim 1, wherein the monitoring step is by a method selected from the group consisting of co-precipitation, protein interactive trapping and ELISA.

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GAGGATCTAC AGGGGACAAG TAAAGGCTAC ATCCAGATGC CGGGAATGCA CTGACGCCCA
    60

TTCCTGGAAA CTGGGCTCCC ACTCAGCCCC TGGGAGCAGC AGCCGCCAGC CCCTCGGACC
   120

TCCATCTCCA CCCTGCTGAG CCACCCGGGT TGGGCCAGGA TCCCGGCAGG CTGATCCCGT
   180

CCTCCACTGA GACCTGAAAA ATG GCT TCG GGG CAA GGC CCA GGT CCT CCC
   230
                      Met Ala Ser Gly Gln Gly Pro Gly Pro Pro
                        1               5                  10

AGG CAG GAG TGC GGA GAG CCT GCC CTG CCC TCT GCT TCT GAG GAG CAG
   278
Arg Gln Glu Cys Gly Glu Pro Ala Leu Pro Ser Ala Ser Glu Glu Gln
                 15                  20                  25

GTA GCC CAG GAC ACA GAG GAG GTT TTC CGC AGC TAC GTT TTT TAC CGC
   326
Val Ala Gln Asp Thr Glu Glu Val Phe Arg Ser Tyr Val Phe Tyr Arg
             30                  35                  40

CAT CAG CAG GAA CAG GAG GCT GAA GGG GTG GCT GCC CCT GCC GAC CCA
   374
His Gln Gln Glu Gln Glu Ala Glu Gly Val Ala Ala Pro Ala Asp Pro
         45                  50                  55

GAG ATG GTC ACC TTA CCT CTG CAA CCT AGC AGC ACC ATG GGG CAG GTG
   422
Glu Met Val Thr Leu Pro Leu Gln Pro Ser Ser Thr Met Gly Gln Val
     60                  65                  70

GGA CGG CAG CTC GCC ATC ATC GGG GAC GAC ATC AAC CGA CGC TAT GAC
   470
Gly Arg Gln Leu Ala Ile Ile Gly Asp Asp Ile Asn Arg Arg Tyr Asp
 75                  80                  85                  90

TCA GAG TTC CAG ACC ATG TTG CAG CAC CTG CAG CCC ACG GCA GAG AAT
   518
Ser Glu Phe Gln Thr Met Leu Gln His Leu Gln Pro Thr Ala Glu Asn
                 95                 100                 105

GCC TAT GAG TAC TTC ACC AAG ATT GCC ACC AGC CTG TTT GAG AGT GGC
   566
Ala Tyr Glu Tyr Phe Thr Lys Ile Ala Thr Ser Leu Phe Glu Ser Gly
             110                 115                 120
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Fig. 1

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ATC AAT TGG GGC CGT GTG GTG GCT CTT CTG GGC TTC GGC TAC CGT CTG
   614
Ile Asn Trp Gly Arg Val Val Ala Leu Leu Gly Phe Gly Tyr Arg Leu
        125                 130                 135

GCC CTA CAC GTC TAC CAG CAT GGC CTG ACT GGC TTC CTA GGC CAG GTG
   662
Ala Leu His Val Tyr Gln His Gly Leu Thr Gly Phe Leu Gly Gln Val
    140                 145                 150

ACC CGC TTC GTG GTC GAC TTC ATG CTG CAT CAC TGC ATT GCC CGG TGG
   710
Thr Arg Phe Val Val Asp Phe Met Leu His His Cys Ile Ala Arg Trp
155                 160                 165                 170

ATT GCA CAG AGG GGT GGC TGG GTG GCA GCC CTG AAC TTG GGC AAT GGT
   758
Ile Ala Gln Arg Gly Gly Trp Val Ala Ala Leu Asn Leu Gly Asn Gly
                175                 180                 185

CCC ATC CTG AAC GTG CTG GTG GTT CTG GGT GTG GTT CTG TTG GGC CAG
   806
Pro Ile Leu Asn Val Leu Val Val Leu Gly Val Val Leu Leu Gly Gln
            190                 195                 200

TTT GTG GTA CGA AGA TTC TTC AAA TCA TGACTCCCAA GGGTGCCCTT
   853
Phe Val Val Arg Arg Phe Phe Lys Ser
        205                 210

TGGGTCCCGG TTCAGACCCC TGCCTGGACT TAAGCGAAGT CTTTGCCTTC TCTGTTCCCT
   913

TGCAGGGTCC CCCCTCAAGA GTACAGAAGC TTTAGCAAGT GTGCACTCCA GCTTCGGAGG
   973

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  1033

TCTCTCTGGG CCCAGGGCTG TGGCCGTCTC CTCCCTCAGC TCTCTGGGAC CTCCTTAGCC
  1093

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  1153

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1333

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1393

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1453

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1513

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1573

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CCCCACCCTC CATGCTCCTC ACCTGTCTAG GTCTCCTCAG GGTGGGGGGT GACAGTGCCT
1933

TCTCTATTGG CACAGCCTAG GGTCTTGGGG GTCAGGGGGG AGAAGTTCTT GATTCAGCCA
1993

AATGCAGGGA GGGGAGGCAG ATGGAGCCCA TAGGCCACCC CCTATCCTCT GAGTGTTTGG
2053

AAATAAACTG TGCAATCCCC TCAAAAAAAA AACGGAGATC C
2094

```
          10          20          30          40          50          60
           *           *           *           *           *           *
TTT TAA TAT AAA TTA ATG TGC TCT ATT TAT AGA GAC AAT ACA TGA AAT ATA CTT AAT AAA
AAA ATT ATA TTT AAT TAC ACG AGA TAA ATA TCT CTG TTA TGT ACT TTA TAT GAA TTA TTT

          70          80          90         100         110         120
           *           *           *           *           *           *
AAT TCA AAT GTT ATA GAA CTG AAA AAG ATG AAA AGT AAA AAC AAC CTA TTC CCC AGA GGT
TTA AGT TTA CAA TAT CTT GAC TTT TTC TAC TTT TCA TTT TTG TTG GAT AAG GGG TCT CCA

         130         140         150         160         170         180
           *           *           *           *           *           *
AGC CAC TGT CCA TAG TTT CTA TTT TAG ATT CTT TCC TTT ATA CAA GAT TAT TAT AGC TTC
TCG GTG ACA GGT ATC AAA GAT AAA ATC TAA GAA AGG AAA TAT GTT CTA ATA ATA TCG AAG

         190         200         210         220         230         240
           *           *           *           *           *           *
TAT TTT TTG GTG TAT GAA CTG TAG TCC TAG AGG ATT TTA TTA GTT ATG AGT TCT ATA ACT
ATA AAA AAC CAC ATA CTT GAC ATC AGG ATC TCC TAA AAT AAT CAA TAC TCA AGA TAT TGA

         250         260         270         280         290         300
           *           *           *           *           *           *
AAG ATC CAT CAT CTT AGT TGC TAA GAA CGT AGA TAC TGA GAA CAT CAT TTA AAA AAA CAT
TTC TAG GTA GTA GAA TCA ACG ATT CTT GCA TCT ATG ACT CTT GTA GTA AAT TTT TTT GTA

         310         320         330         340         350         360
           *           *           *           *           *           *
TTT TGG CTG GCA CCT CAT GAT CAC TGG AGT CTC GCG GGT CCC TCA GGC TGC ACA GGG ACA
AAA ACC GAC CGT GGA GTA CTA GTG ACC TCA GAG CGC CCA GGG AGT CCG ACG TGT CCC TGT

         370         380         390         400         410         420
           *           *           *           *           *           *
AGT AAA GGC TAC ATC CAG ATG CTG GGA ATG CAC TGA CGC CCA TTC CTG GAA ACT GGG CTC
TCA TTT CCG ATG TAG GTC TAC GAC CCT TAC GTG ACT GCG GGT AAG GAC CTT TGA CCC GAG

         430         440         450         460         470         480
           *           *           *           *           *           *
CCA CTC AGC CCC TGG GAG CAG CAG CCG CCA GCC CCT CGG GAC CTC CAT CTC CAC CCT GCT
GGT GAG TCG GGG ACC CTC GTC GTC GGC GGT CGG GGA GCC CTG GAG GTA GAG GTG GGA CGA

         490         500BamHI    510         520         530         540
           *           *|          *           *           *           *
GAG CCA CCC GGG TTG GGC CAG GAT CCC GGC AGG CTG ATC CCG TCC TCC ACT GAG ACC TGA
CTC GGT GGG CCC AAC CCG GTC CTA GGG CCG TCC GAC TAG GGC AGG AGG TGA CTC TGG ACT

         550         560         570         580         590         600
           *           *           *           *           *           *
AAA ATG GCT TCG GGG CAA GGC CCA GGT CCT CCC AGG CAG GAG TGC GGA GAG CCT GCC CTG
TTT TAC CGA AGC CCC GTT CCG GGT CCA GGA GGG TCC GTC CTC ACG CCT CTC GGA CGG GAC
     M   A   S   G   Q   G   P   G   P   P   R   Q   E   C   G   E   P   A   L>

         610         620         630         640         650         660
           *           *           *           *           *           *
CCC TCT GCT TCT GAG GAG CAG GTA GCC CAG GAC ACA GAG GAG GTT TTC CGC AGC TAC GTT
GGG AGA CGA AGA CTC CTC GTC CAT CGG GTC CTG TGT CTC CTC CAA AAG GCG TCG ATG CAA
  P   S   A   S   E   E   Q   V   A   Q   D   T   E   E   V   F   R   S   Y   V>

         670         680         690         700         710         720
           *           *           *           *           *           *
TTT TAC CAC CAT CAG CAG GAA CAG GAG GCT GAA GGG GCG GCT GCC CCT GCC GAC CCA GAG
AAA ATG GTG GTA GTC GTC CTT GTC CTC CGA CTT CCC CGC CGA CGG GGA CGG CTG GGT CTC
  F   Y   H   H   Q   Q   E   Q   E   A   E   G   A   A   A   P   A   D   P   E>
```

Fig. 2

```
          730          740          750 >Nco1    760          770          780
           *            *            *    |       *            *            *
ATG GTC ACC TTA CCT CTG CAA CCT AGC AGC ACC ATG GGG CAG GTG GGA CGG CAG CTC GCC
TAC CAG TGG AAT GGA GAC GTT GGA TCG TCG TGG TAC CCC GTC CAC CCT GCC GTC GAG CGG
 M   V   T   L   P   L   Q   P   S   S   T   M   G   Q   V   G   R   Q   L   A>

          790          800          810          820          830          840
           *            *            *            *            *            *
ATC ATT GGG GAC GAC ATC AAC CGA CGC TAT GAC TCA GAG TTC CAG ACC ATG TTG CAG CAC
TAG TAA CCC CTG CTG TAG TTG GCT GCG ATA CTG AGT CTC AAG GTC TGG TAC AAC GTC GTG
 I   I   G   D   D   I   N   R   R   Y   D   S   E   F   Q   T   M   L   Q   H>

   >Pst1  850          860          870          880          890          900
     |     *            *            *            *            *            *
CTG CAG CCC ACG GCA GAG AAT GCC TAT GAG TAC TTC ACC AAG ATT GCC TCC AGC CTG TTT
GAC GTC GGG TGC CGT CTC TTA CGG ATA CTC ATG AAG TGG TTC TAA CGG AGG TCG GAC AAA
 L   Q   P   T   A   E   N   A   Y   E   Y   F   T   K   I   A   S   S   L   F>

          910          920          930          940          950          960
           *            *            *            *            *            *
GAG AGT GGC ATC AAT TGG GGC CGT GTG GTG GCT CTT CTG GGC TTC AGC TAC CGT CTG GCC
CTC TCA CCG TAG TTA ACC CCG GCA CAC CAC CGA GAA GAC CCG AAG TCG ATG GCA GAC CGG
 E   S   G   I   N   W   G   R   V   V   A   L   L   G   F   S   Y   R   L   A>

          970          980          990         1000         1010         1020
           *            *            *            *            *            *
CTA CAC ATC TAC CAG CGT GGC CTG ACT GGC TTC CTG GGC CAG GTG ACC CGC TTT GTG GTG
GAT GTG TAG ATG GTC GCA CCG GAC TGA CCG AAG GAC CCG GTC CAC TGG GCG AAA CAC CAC
 L   H   I   Y   Q   R   G   L   T   G   F   L   G   Q   V   T   R   F   V   V>

         1030         1040         1050         1060         1070         1080
           *            *            *            *            *            *
GAC TTC ATG CTG CAT CAC TGC ATT GCC CGG TGG ATT GCA CAG AGG GGT GGC TGG GTG GCA
CTG AAG TAC GAC GTA GTG ACG TAA CGG GCC ACC TAA CGT GTC TCC CCA CCG ACC CAC CGT
 D   F   M   L   H   H   C   I   A   R   W   I   A   Q   R   G   G   W   V   A>

         1090         1100         1110         1120         1130         1140
           *            *            *            *            *            *
GCC CTG AAC TTG GGC AAT GGT CCC ATC CTG AAC GTG CTG GTG GTT CTG GGT GTG GTT CTG
CGG GAC TTG AAC CCG TTA CCA GGG TAG GAC TTG CAC GAC CAC CAA GAC CCA CAC CAA GAC
 A   L   N   L   G   N   G   P   I   L   N   V   L   V   V   L   G   V   V   L>

         1150         1160         1170         1180         1190         1200
           *            *            *            *            *            *
TTG GGC CAG TTT GTG GTA CGA AGA TTC TTC AAA TCA TGA CTC CCA AGG GTG CCT TTG GGG
AAC CCG GTC AAA CAC CAT GCT TCT AAG AAG TTT AGT ACT GAG GGT TCC CAC GGA AAC CCC
 L   G   Q   F   V   V   R   R   F   F   K   S   *>

         1210         1220         1230         1240         1250         1260
           *            *            *            *            *            *
TCC CAG TTC AGA CCC CTG CCT GGA CTT AAG CGA AGT CTT TGC CTT CTC TGC TCC TTG CAG
AGG GTC AAG TCT GGG GAC GGA CCT GAA TTC GCT TCA GAA ACG GAA GAG ACG AGG AAC GTC

         1270         1280 Hind3
           *            *    |
GT CCC CCC TCA AGA GTA CAG AAG CTT
CCA GGG GGG AGT TCT CAT GTC TTC GAA
```

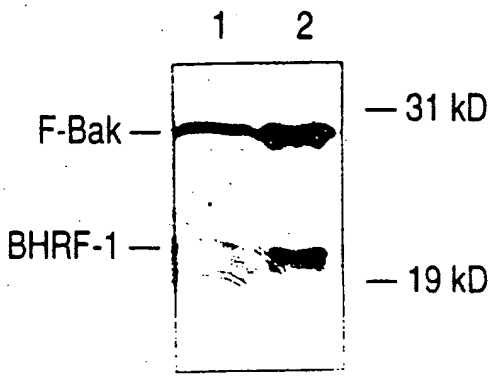

FIG. 3A

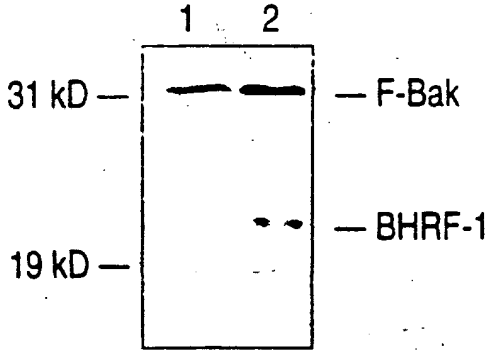

FIG. 3B

INTERNATIONAL SEARCH REPORT

International Application No
PC./US 96/05639

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 C07K14/47 C07K14/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,95 05738 (MASSACHUSETTS INST TECHNOLOGY) 2 March 1995 Adenovirus E1B protein see page 10, line 1 - page 10, line 7 ---	1-6
A	CANCER RES. (1994), 54(10), 2808-11 CODEN: CNREA8;ISSN: 0008-5472, 1994, XP002007901 HICKISH, TAMAS ET AL: "Ultrastructural localization of BHRF1: an Epstein-Barr virus gene product which has homology with bcl-2" see the whole document --- -/--	1-6

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 July 1996	03. 10. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hoekstra, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VIROLOGY (1994), 201(2), 404-7 CODEN: VIRLAX;ISSN: 0042-6822, 1994, XP002007902 TARODI, BELA ET AL: "Epstein-Barr virus BHRF1 protein protects against cell death induced by DNA-damaging agents and heterologous viral infection" see the whole document ---	1-6
A	WO,A,95 05750 (UNIV WASHINGTON ;KORSMEYER STANLEY J (US)) 2 March 1995 see claim 8 ---	1-6
A	CELL, vol. 74, August 1993, page 609-619 XP002007903 OLTVAI, Z.N. ET AL.: "Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death" see the whole document ---	1-6
P,X	NATURE, vol. 374, no. 6524, 20 April 1995, pages 731-733, XP002007904 FARROW, S.N.: "cloning of a bcl-2 homologue by interaction with adenovirus E1B 19K" see page 733, left-hand column, last paragraph ---	1-6
P,A	NATURE, vol. 374, 20 April 1996, pages 736-739, XP002007905 MICHAEL J. KIEFER ET AL.: "Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak" see page 739, right-hand column ---	1-6
P,A	WO,A,95 15084 (LXR BIOTECHNOLOGY INC ;KIEFER MICHAEL C (US); BARR PHILIP J (US)) 8 June 1995 cited in the application see page 16, line 25 - line 31 -----	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/05639

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9505738	02-03-95	NONE		
WO-A-9505750	02-03-95	AU-B-	7604994	21-03-95
		CA-A-	2170143	02-03-95
WO-A-9515084	08-06-95	AU-B-	1335195	19-06-95